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# Non-Contact Microdrilling of Mouse Zona Pellucida With an Objective-Delivered 1.48-μm Diode Laser

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***Background and Objective:*** **A non-touch laser-induced microdrilling procedure is studied on mouse zona pellucida (ZP).**
***Study Design/Materials and Methods:*** **A 1.48-μm diode laser beam is focused in a 8-μm spot through a 45× objective of an inverted microscope. Mouse zygotes, suspended in a culture medium, are microdrilled by exposing their ZP to a short laser irradiation and allowed to develop in vitro.**
***Results:*** **Various sharp-edged holes can be generated in the ZP with a single laser irradiation. Sizes can be varied by changing irradiation time (3–100 ms) or laser power (22–55 mW). Drilled zygotes present no signs of thermal damage under light and scanning electron microscopy and develop as expected in vitro, except for a distinct eight-shaped hatching behavior.**
***Conclusion:*** **The microdrilling procedure can generate standardized holes in mouse ZP, without any visible side effects. The hole formation can be explained by a local photothermolysis of the protein matrix.** 

**Key words: assisted fertilization, infrared laser, microdissection, oocyte, photothermolysis**

## INTRODUCTION

Since laser discovery in the early 1960s, many medical applications of this technology have been proposed. However, most of them did not take full advantage of the remarkable properties of laser light, in particular its huge spatial focusing potential. Despite the early recognition of the possible actions of lasers at a microscopic level [1], cellular or subcellular surgery with laser light remained mostly unexplored until the 1980s [2]. Since then an increasing number of laser applications at the cellular level have been reported, such as cell fusion [3] or gene partial destruction [4].

Fertilization and implantation in mammals is controlled by the zona pellucida (ZP), a thick protein coating surrounding the ooplasm, which has to be crossed by the male gamete before fertilization can take place and which has to be broken when the blastocyst is able to implant. Male infertility can be related in some circumstances to the inability of the spermatozoa to accomplish this essential step. Since the mid-1980s several methods have been proposed to circumvent this functional defect by creating an aperture in the ZP chemically [5] or mechanically, by partial zona dissection [6] or slitting [7]. A similar procedure has also been proposed to treat cases for which an

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inability of the embryo to implant was linked to a hatching failure [8]. These methods have the main drawbacks of being technically complex and unable to produce standardized holes. At the fertilization time, a slit that is too large may increase the rate of polyspermic fertilization [9]; later on, the implantation ability can be altered by a loss of material [8,10] or the breakage of immunological barriers [11]. In contrast, lasers are potentially ideal tools to drill holes of improved size and quality. Laser-assisted zona drilling was first reported by two groups [12,13]. Palanker et al. reported successful in vitro fertilization and blastocyst formation of mouse oocytes drilled with an 193-nm ArF excimer laser [13]; however, the very low penetration depth of the 193 nm UV radiation in the culture medium (less than 1 μm) made the use of a micropipette necessary for the delivery of the laser light close to the egg ZP. Blanchet et al. experimented with 248-nm KrF excimer radiation; they were able to drill cryopreserved two-cell mouse embryos with objective-delivered laser light [14]. Neev et al. [15] investigated other wavelengths (266, 308, 355, 366, and 532 nm), which are less absorbed by the culture medium and allow a non-touch objective-delivery of the laser light. In this comparative study, they concluded that the 308-nm emission of the XeCl laser was the best suited for ZP microdrilling and demonstrated improved in vitro fertilization rates by spermatozoa of long-term vasectomized mice after laser drilling of oocytes [16]. Among the UV lasers, a commercially available 332-nm nitrogen laser has been proposed in combination with optical tweezers as a micromanipulation tool for the enhancement of in vitro fertilization [17]. Feichtinger et al. [18] introduced the strongly water-absorbed 2.94-μm Er: YAG radiation for assisted in vitro fertilization in humans. The important advantage of this laser is that its wavelength is less likely to induce the unwanted mutagenic side effects that have been described for the UV region [19]. As this radiation is strongly absorbed by water, the delivery of the 2.94-μm radiation could not be performed through a microscope objective and was only possible through an optical fiber brought in immediate contact with the ZP [20]. The first human pregnancies obtained after ZP drilling with this technique have been reported [18,21]. Recently, the intracytoplasmic sperm injection (ICSI) using micropipettes has been proposed as an alternative and promising tool in respect to partial zona dissection (PZD) [22] and subzonal insemination (SUZI) [23]. Future safety assessment of these techniques is needed to fully appreciate the advantages/disadvantages of these approaches.

The purpose of this study is to investigate another wavelength of the infrared spectrum, produced by a small and cost-effective diode laser. The investigated 1.48-μm wavelength is less absorbed by water than the 2.94 μm wavelength and can thus be focused through microscope objective and culture dishes at the level of subcellular components. The demonstration that this radiation is able to generate openings in the ZP of mouse zygotes [24] has prompted a more careful study on the physico-chemical mechanisms involved in this process and on the impact of the procedure on early embryogenesis.

## MATERIALS AND METHODS

### Experimental Set-Up

The experimental set-up used for ZP microdrilling is described in Figure 1. The InGaAsP laser diode (Alcatel/Alsthom Research, France), emitting at a wavelength of 1.48 μm, has a single stripe structure (stripe length: 605 μm) and an emitting area of $2 \times 0.2$ μm$^2$ (M. Matabon, Alcatel/Alsthom Research, private communication). The physical dimensions of the diode laser are $9 \times 5 \times 5$ mm$^3$. The broad band laser emission (15 nm) is monomode transversal. The diode laser beam divergence is $19 \pm 1$ degrees and $23 \pm 2$ degrees, respectively, in the directions parallel and perpendicular to the junction. In cw-operation, a laser power up to 120 mW is obtained for a diode current of 350 mA. The invisible 1.48-μm diode laser beam is first collimated through a microscope objective ($L_1$), corrected for a wavelength of 1.5 μm, with a 4.4-mm focal length and a 0.65 N.A. (Projectina, type 190-9004, Switzerland). It is then matched to a 1-mW visible 670-nm aiming diode laser beam with the mirror $M_1$; $M_1$ has a high reflectivity for the 1.48-μm radiation and is semi-transparent for the 670-nm radiation of the aiming beam. The collinear 1.48-μm and 670-nm laser beams are coupled to the inverted microscope (Biostar, Leica Inc., USA) by a set of two mirrors ($M_2$ and $M_3$) and lens $L_2$. The two collinear laser beams are directed along the microscope optical axis by $M_3$ (high reflectivity at 1.48 μm, high transmission in the visible and semi-transparent at 670 nm). $L_2$ (focal length: 105 mm) allows focusing of the incident 1.48-μm and 670-nm beams on the image plane of the 45× microscope objective $L_3$. The spot size of the laser beam in the

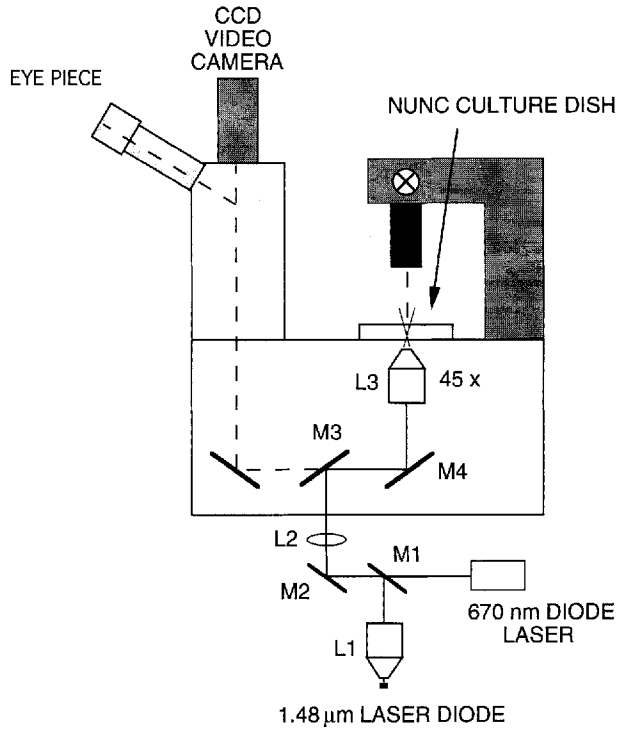


Fig. 1. Experimental arrangement. The collimated 1.48-μm diode laser beam is coupled to the inverted microscope by mirrors $M_1$, $M_2$, and $M_3$ and an additional focusing lens $L_2$. It is then focused in a 8-μm spot by the microscope objective $L_3$.

object plane has been carefully measured with the knife-edge method [25]. The measured spot diameter is 8 ± 0.5 μm. Transmission of the coupling optics has been determined with a power meter (Coherent model 210). A maximal laser power of 55 mW, corresponding to 48% of the 120 mW laser output, is available at the exit of $L_3$. This corresponds to a power density of 109 kW/cm$^2$ at the object plane. For most experiments, however, the output power has been limited to 47 mW.

### Absorption Coefficient Measurement

Absorption coefficients of the 1.48-μm laser beam by the culture medium or demineralized water were derived directly from extinction measurements through fluid depths ranging between 1.5–3.0 mm.

### Egg Collection and Culture

Mouse zygotes were obtained 20 h after mating from 5- to 8-week-old females (B6D2F1, IFFA CREDO, France) following a standard superovulation procedure [26]. Collected eggs were rinsed and suspended in human tubal fluid medium (HTF) [27], containing 0.5% serum albumin (WBAG, Zürich, Switzerland). Control zygotes were suspended in groups of 15–20 in 1 ml $HCO_3^-$-buffered HTF (HTF-$HCO_3^-$) in four-well multidishes (Nunclon, Gibco, Basel, Switzerland) and were immediately incubated under standard conditions (37°C, 5% $CO_2$, 5% $O_2$, 90% $N_2$). Treated zygotes were separated in groups of five to ten in 0.5 ml HEPES-buffered HTF (HTF-HEPES), covered with 0.2 ml mineral oil (Light mineral oil, Fisher, USA) in four-well multidishes, and kept at a temperature of 37°C until laser treatment. After the drilling procedure, zygotes were transferred in fresh HTF-$HCO_3^-$ medium and allowed to grow up to 5–6 days under the standard conditions. Occurrence of morula, blastocysts and hatching were recorded and documented by photographs. Diameters and thicknesses of empty ZP were measured on photographs.

Unfertilized oocytes, used in some experiments, were isolated from superovulated females 14 h after ovulation induction and processed according to the same procedure as that used for zygotes.

### Drilling Procedure

The culture dish was placed on the displacement stage of the microdrilling set-up (Fig. 1), and the group of eggs was located first at low magnification. For the drilling procedure, the 45× objective was selected. The egg ZP was positioned with the displacement stage on the control aiming spot and exposed to laser light (2–1,000 ms; 0.1–22 mJ). In order to minimize interactions of the laser beam with the egg cytoplasm, the aiming spot was brought onto a region of the zona where the perivitelline space (PVS) was widest.

Two drilling sites have been studied. In the first configuration (Fig. 2a), the egg was placed tangentially to the diode laser beam in order to induce a trench in the ZP. A complete opening or a local thinning of the ZP could be generated at will by the precise positioning of the laser focalization point within the ZP width. Even when ZP opening was achieved, most of the laser radiation was confined outside the egg. In the second mode (Fig. 2b), the egg was placed in such a way that the laser beam intersected the ZP in a more poleward location. In this configuration, the laser beam passed through a portion of the PVS. Direct interaction of the laser beam with the cytoplasm was, however, avoided. Using this irradiation configuration, cylindrical holes could be generated in the ZP.

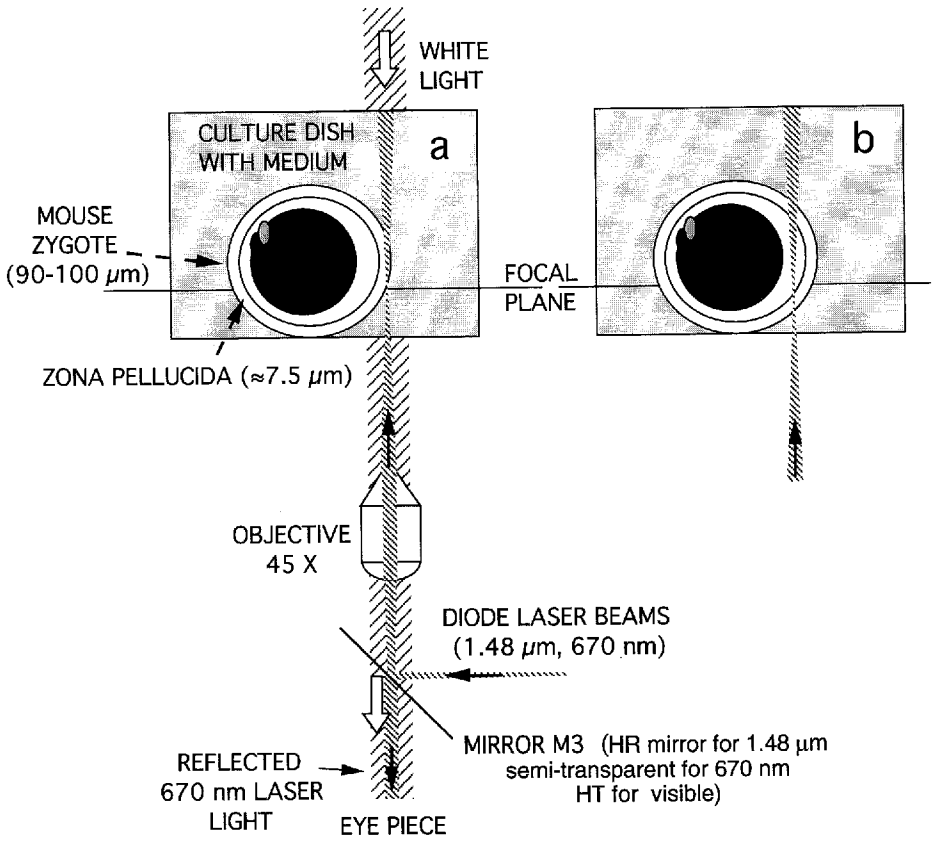


Fig. 2. Laser drilling procedure. The ZP is positioned with the displacement stage of the microscope. In order to reach the maximal drilling efficiency, the laser localization point is placed at the surface of the zona. Two procedures have been tested. **a:** Generation of a trench by tangential positioning of the zona. **b:** Drilling of the zona by placing the laser impact point on the zygote in an inner location without intersection with the cytoplasm.

No micromanipulators were needed to stabilize the eggs during the drilling procedure in the standard conditions. Egg movements due to convective flows within the culture medium were observed only when very long irradiation times (>100 ms) were used. The drilling procedure was monitored and systematically recorded with a CCD video camera. Hole sizes were determined using a calibrated scale displayed on the video monitor. The drilling threshold for a given laser output was determined by the shortest irradiation time necessary to produce a visible hole in the ZP at the working magnification.

### Scanning Electron Microscopy

Eggs were drilled on four opposite locations in order to increase the number of observable sites. After drilling, the eggs were transferred in 1% glutaraldehyde, buffered with cacodylate (pH 7.4), fixed for about 2 h, and rinsed in cacodylate buffer at 4°C. Round coverslips (11 mm diameter) were washed with acidic ethanol, covered with L-polylysin (MW ≥ 300,000), and air-dried. A hydrophobic circle was marked (Dako Pen, S2002, Dako, USA) to prevent spreading of the buffer, when eggs were placed on the slide. After a 48-hr incubation time (4°C, under moist atmosphere), the slides were dehydrated in graduated series of ethanol and dried using the critical point method. The specimens were finally vacuum-coated with platinum and examined using a JEOL 6300 F scanning electron microscope. Dimensions observed under scanning electron microscopy did not represent those of the eggs and drilled holes before preparation, as the dehydration procedure led to a shrinkage of the specimens.

## RESULTS

### Absorption of the 1.48-μm Diode Laser Beam in Water and Culture Medium

The measured absorption coefficients for demineralized water and the culture medium (HTF-HEPES) were $\mu_{a_{H_2O}} = 28.1\ cm^{-1}$ and $\mu_{a_{HTF}HEPES} =$

26.3 $cm^{-1}$. These values represent 1/e penetration depths of 356 μm in demineralized water and 380 μm in the culture medium. Taking these figures into account and assuming an egg diameter of 100 μm, the calculated attenuation of the laser beam by the culture medium varies from 5 to 12% for ZP impact locations situated 20–50 μm from the bottom of the dish (Fig. 2a,b). As the laser attenuation could not be determined precisely for each individual egg, the laser irradiance values reported in this work were not corrected for medium absorption.

### Zona Pellucida Drilling

Holes could be drilled with the 1.48-μm diode laser for laser powers ranging from 22 to 55 mW and for irradiation times of 2–1,000 ms with a single laser irradiation. For irradiation times greater than the frame rate of the video recording (25 ms), the progression of the drilling process and growth of the drilled channel could be followed on subsequent video frames. The drilled channel was seen to grow continously while small particles were flowing away from the laser impact region. At each investigated laser power, a series of holes was drilled using increasing irradiation times. An example of such a series, performed on a zygote at a laser power of 47 mW and an estimated power density of 94 $kW/cm^2$, is shown on Figure 3. The smallest drilled hole (2-μm diameter, upper left) has been obtained with a 5-ms irradiation time, i.e., a total energy of 235 μJ, and the largest (12 μm, upper right) with a 30-ms irradiation time (1,410 μJ).

The hole diameters as a function of the irradiation time have been studied using fertilized (Fig. 4) and unfertilized eggs (Fig. 5) for a laser power of 22–55 mW, i.e., a laser irradiance of 44–109 $kW/cm^2$. For constant irradiation conditions, a hole could be drilled reproducibly with less than 1 μm variation in their diameter. For each laser irradiance, a logarithmic relationship between the hole diameter and the irradiation time (which is directly related to the deposited energy) was observed. As expected, the drilling threshold decreased with increasing laser irradiance.

In case of fertilized eggs (zygotes), the drilling threshold measured at the maximal available laser power of 55 mW (109 $kW/cm^2$) was less than 2 ms, corresponding to a total deposited energy of less than 110 μJ (Fig. 4). The resulting 2-μm-wide hole had the shape of a crater extending only into a portion of the irradiated ZP thickness. When the laser power was reduced to 22 mW (44 $kW/cm^2$),

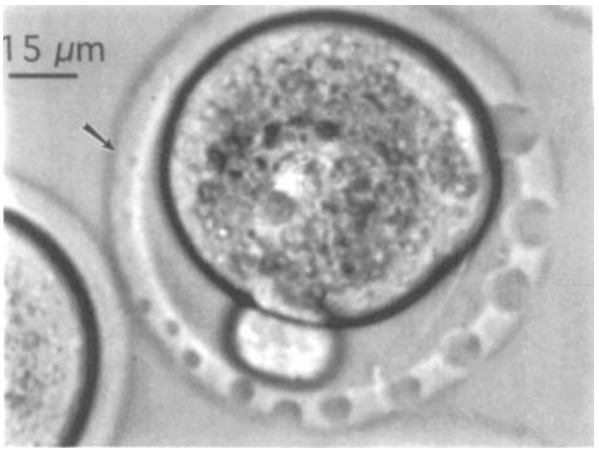


Fig. 3. Series of holes drilled in a mouse zygote ZP for increasing irradiation times. Laser power: 47 mW, irradiance: 94 $kW/cm^2$, irradiation times: 5 ms (arrow, upper left) to 30 ms (upper right).

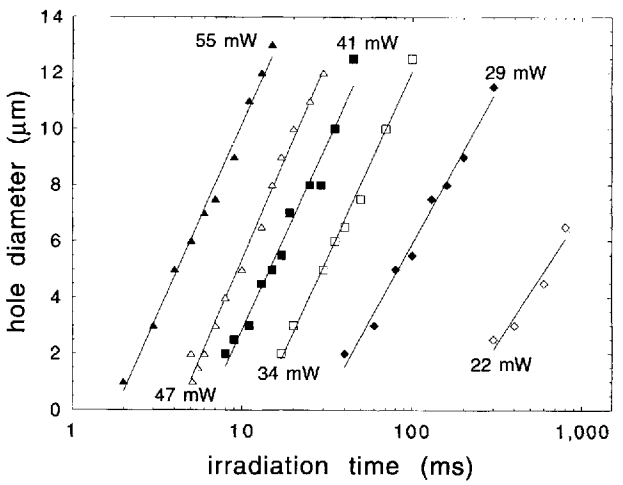


Fig. 4. Diameter of holes drilled in the ZP of mouse zygotes as a function of the irradiation time for different laser powers.

the drilling threshold was increased to 200 ms. In order to obtain hole diameters of 5–7 μm, 4- to 6-ms irradiation times were necessary at 55 mW, corresponding to a total delivered laser energy of 225–330 μJ. For the same laser irradiance, the largest holes (13 μm diameter) were obtained with an irradiation time of 15 ms (825 μJ).

A similar overall picture was observed in the case of unfertilized eggs (oocytes), except that the total laser energies needed to drill a hole of a given size were typically on the order of 20% lower than those needed for zygotes. Hole diameters of 5–7 μm, large enough for a spermatozoon to enter, were drilled in 3.5–4.7 ms at 55 mW (109

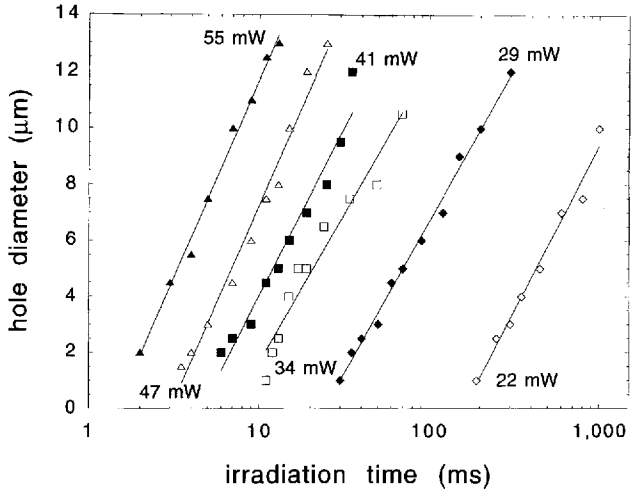


Fig. 5. Diameter of holes drilled in the ZP of mouse oocytes as a function of the irradiation time for different laser powers.

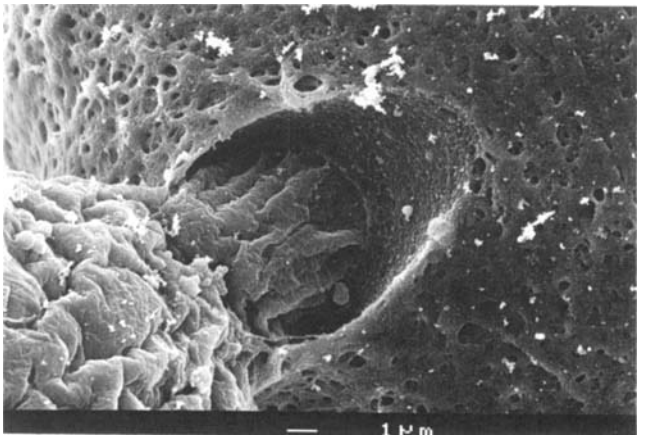

Fig. 6. Scanning electron micrograph of a hole drilled in a frozen-thawed zygote with the 1.48-µm laser diode at a power of 47 mW and an irradiation time of 15 ms. Note the sharp wall drilled in the ZP. The protuberant cytoplasmic material is due to the preparation artifact. Hole size is smaller than normal owing to the electron microscopy preparation.

kW/cm$^2$), corresponding to laser energy doses of 190-260 µJ.

### Hole Characteristics

Quality of the holes obtained at a laser output power of 47 mW and short irradiation times has been assessed by scanning electron microscopy. A typical hole drilled in the ZP of a frozen-thawed zygote is shown in Figure 6. At the 94-kW/cm$^2$ irradiance used, an irradiation time of 15 ms, corresponding to an energy of 705 µJ, was needed to drill the hole. The irradiation mode was selected according to the technique described in Figure 2b. The high quality of the drilling procedure is demonstrated by the shape of the drilled channel which appears as a perfect cylinder with a smooth surface and regular incision edges throughout the ZP. At the surface of the ZP, the border of the drilled hole is clearly delimited and there is no evidence for a transitory alteration of the ZP matrix in the immediate vicinity of the channel. The fact that the irradiation axis does not cross the center of the egg sphere is recognized by the ellipsoid shape of the surface aperture. Note that the protuberant cytoplasmic material from the drilled hole is an artifact of the preparation technique for scanning electron microscopy.

Figure 7 is an example of a trench generated by a tangential laser ablation using the irradiation mode described in Figure 2a. The ablation parameters were an irradiance of 94 kW/cm$^2$ (laser power: 47 mW) and an irradiation time of 20 ms (total energy: 940 µJ). Access or not to the perivitelline space (PVS) can be selected by varying the trench diameter or the laser impact within the ZP width, as shown on Figures 8 and 7b. On Figure 8, the ooplasm can be seen in the center of the trench (see arrow). By choosing the laser impact point according to the irradiation modes described in Figure 2, it is thus possible to generate either a cylindrical aperture in the zona with access to the PVS or a trench reaching or not reaching the PVS.

### In Vitro Development After ZP Drilling

In order to assess the impact of the laser drilling procedure, in vitro development of drilled zygotes has been compared to that of appropriate controls. Various developmental stages of control and drilled embryos are shown in Figure 9. The drilling procedure did not affect the early cleavages and morula compaction up to day 4. The blastocyst formation rates, expressed as blastocyst/initial two-cell embryo (%), were close to 70% in the two groups (Table 1). At day 5, drilled embryos initiated hatching out of the ZP through the drilled hole leading to a characteristic eight-shaped twin blastocyst (Fig. 9c,e). By that time, the control blastocysts were still entrapped in the ZP, which was undergoing enlargement and thinning (Fig. 9d). At day 6, most of the blastocysts had fully hatched away from the ZP in the drilled group, and developmental arrest of partly hatched embryos was rarely observed. In the control group, hatching occurred through a torn portion of the ZP leading to an escape of the blasto-

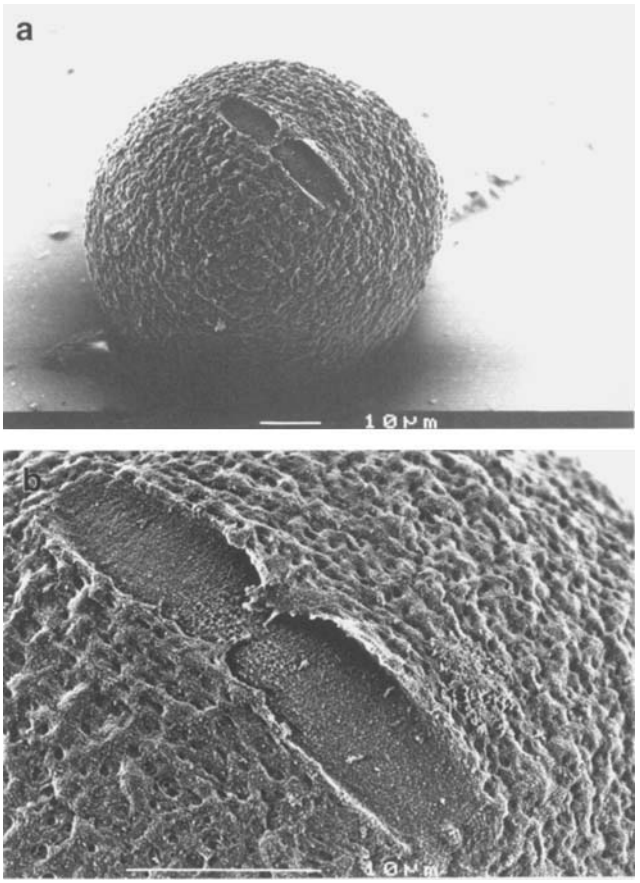


Fig. 7. Scanning electron micrograph of a trench drilled in a mouse oocyte with the 1.48-µm laser diode at a power of 47 mW and an irradiation time of 20 ms. **a:** General view. **b:** Higher magnification of the trench; its diameter corresponds roughly to the thickness of the ZP, but no access to the perivitelline space is visible. Trench size is smaller than normal owing to the electron microscopy preparation.

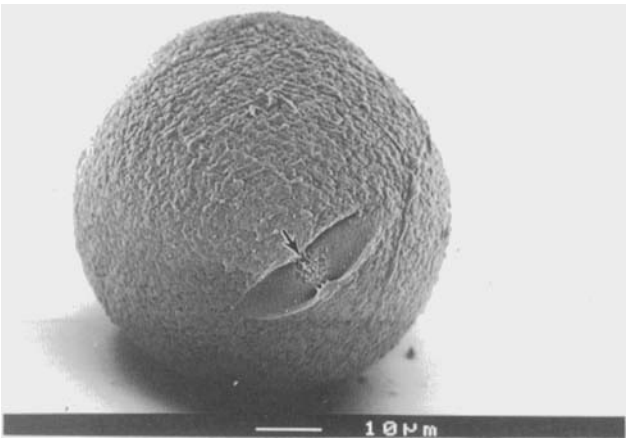


Fig. 8. Scanning electron micrograph of a trench drilled in an oocyte with the same irradiation parameters as in Figure 7. Ooplasm is visible in the center of the trench as the zona has been removed in its total depth (arrow). Trench size is smaller than normal owing to the electron microscopy preparation.

cyst as a whole unit (Fig. 9f). As a consequence, empty ZP were strikingly different in both groups in terms of outer diameter and ZP thickness (Table 1). The ZP outer volume of the control embryos is 1.8 times greater than that of drilled embryos, and their thickness is reduced by a factor of 1.6. Moreover, control ZP exhibited a V-shaped aperture (Fig. 10b, arrow), consistent with a tearing mechanism for ZP opening, whereas drilled ZP presented a round aperture, which clearly derived from the enlargement of the drilled hole (Fig. 10a, arrow).

## DISCUSSION

Compared to other laser drilling procedures, the described set-up, based on the use of a 1.48-µm diode laser, presents several advantages. The absorption coefficient of the 1.48-µm radiation by the culture medium and the culture dish is much lower than those of the 193-nm and 2.94-µm radiation produced by ArF and Er:YAG lasers, respectively; the 1.48-µm laser radiation can thus be focused through the conventional optics of an inverted microscope within a polystyrene culture dish filled with culture medium, providing an easy non-touch and objective-driven access to laser light to specific microscopic cellular subcomponents, such as the ZP of mammalian eggs. Hollow wave guides [13] or optical fibers [18], which were required with the 193-nm and 2.94-µm radiation, are no longer needed. Furthermore, the 1.48-µm radiation can induce specific alterations of the ZP by formation of holes of controllable shapes and sizes. The excellent drilling quality is confirmed by the sharp edges of the drilled holes observed by scanning electron microscopy. Holes of a given diameter can be reproduced with less than 1 µm variation or enlarged by selecting longer irradiation times. The hole diameters increase with the logarithm of the irradiation time for a wide range of irradiance. Holes or trenches can be achieved in a single irradiation of 2–10 ms without any optical readjustment.

When using standard laser irradiation conditions (47 mW, 2–20 ms irradiation times), scanning electron microscopy highlights the fact that the laser damage remains confined to the desired effect on the ZP. Furthermore, the infrared

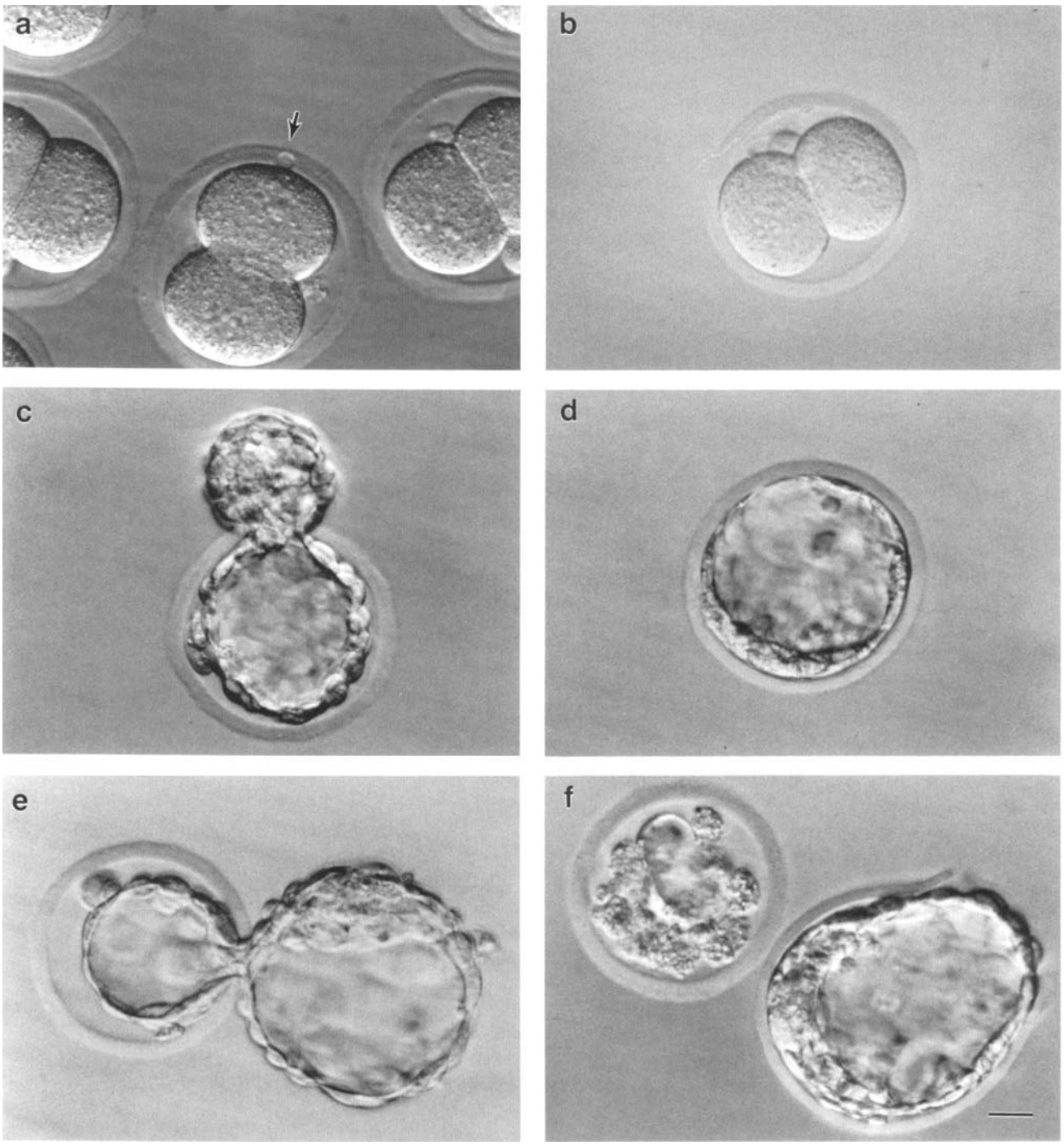


Fig. 9. Developmental stages of mouse control and drilled embryos. Drilled (**a,** see arrow) and control (**b**) two-cell embryos at day 2. **c:** Drilled embryo starting hatching at day 5. **d:** Still entrapped control at day 5. **e:** Eight-shaped drilled embryo at day 6. **f:** Hatching of control at day 6. Note the reduced ZP thickness of the control embryo during hatching at day 6 as compared to control (f, right) as compared to that of a drilled one (e) and to a control that stopped development (f, left). Bar = 20 μm.

1.48-μm procedure has no potential mutagenicity contrary to all UV laser procedures [19]. Zona drilling is performed within the culture medium, thus minimizing the exposition of the sensitive eggs to adverse conditions. As zona opening is obtained quasi-instantaneously, the procedure can

**TABLE 1. Development of Control and Zona-Drilled Embryos In Vitro and Dimensions of Empty ZP After Hatching**

| Groups | Blastocysts/ two-cell embryos | (%) | Hatched blastocysts | (%) | ZP width (μm), mean ± SD | ZP diameter (μm), mean ± SD |
|---|---|---|---|---|---|---|
| Control | 107/152 | (70.4)* | 26/107 | (24.3)** | 5.0 ± 1.0 | 125 ± 4 |
| Laser drilled | 63/90 | (70.0)* | 50/63 | (79.4)** | 8.0 ± 0.5 | 102 ± 2 |

*No statistical differences.
**Statistically different, $P < .0001$.

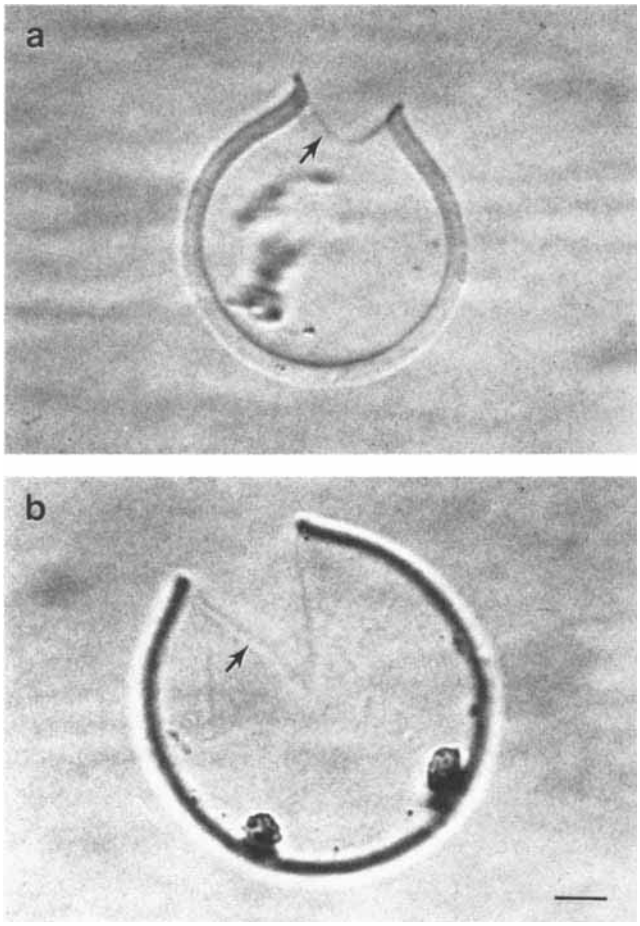


Fig. 10. Examples of empty ZP. **a:** ZP of a drilled zygote exhibiting a round-shaped aperture (arrow) and no thinning. **b:** ZP of control exhibiting thinning, an enlarged outer diameter, and V-shaped tearing (arrow). Bar = 20 μm.

be done by an operator without particular technical skill on a conventional microscope without sophisticated and expensive displacement stages and micromanipulators. In vitro, drilled zygotes exhibited the same aptitude to develop to the blastocyst stage as the controls, except that they started hatching 1 day earlier and went through a temporary figure eight-shaped blastocyst stage before escape from the ZP. This observation is consistent with the fact that the drilled aperture acts as an expansion valve through which the embryonic cells progressively hatch. As a consequence of this peculiar hatching behavior, the ZP thickness and inner volume remain unchanged in drilled embryos, whereas they are significantly modified in the control group. A more complete report on the abiltity of such embryos to implant in vivo can be found elsewhere [28].

As the feasibility and the benefits of the technique are demonstrated, the physical and biophysical bases of the laser interaction process involved must be better understood. The results obtained so far show that the interaction of the well-collimated 1.48-μm cw-laser beam focused to a micron-range spot size on the target can lead to a very localized thermal process. The sharp edges of the drilled holes result from the large irradiance gradient achieved at the laser beam focus. First experimental observations indicate that the material removal process is a lysis of the proteins that constitute the ZP matrix. Indeed neither bubble formation nor a significant acoustic transient could be detected in the vicinity of the drilled eggs. In our opinion a true photothermolysis process is at the origin of the ZP drilling.

The photothermolysis process hypothesized in our experimental conditions is supported by a qualitative estimation of the energy deposition time scale. Let us first determine the characteristic time of heat propagation out of the irradiated volume. The absorption coefficient of the ZP for the 1.48-μm radiation is unknown, but it is probably not much different from that of the surrounding culture medium. We can then assume an absorption length of 380 μm. By contrast the laser spot diameter $\omega$ at the focus is only on the order of 8 μm. Under such conditions, heat is mainly evacuated radially out of the optical zone, and according to the cylindrical geometry of the problem, the heat diffusion characteristic time $\tau$, is given by $\tau = \omega^2/16\kappa$ [26], where $\kappa$ is the thermal diffusivity factor. In our conditions, for a spot diameter of 8 μm and $\kappa \sim 1.5 \times 10^{-3}$ cm$^2$/s for water, $\tau$ is ap-

**TABLE 2. Mouse Zona Pellucida Drilling Parameters of the Laser Systems Described in the Literature***

| Laser | InGaAsP diode (this study) | ArF [13] | KrF [14] | XeCl [15,16][a] | Er:YAG [20][b] |
|---|---|---|---|---|---|
| Wavelength (nm) | 1,480 | 193 | 248 | 308 | 2,940 |
| Pulse duration (s) | $5 \cdot 10^{-3}$ | $1.5 \cdot 10^{-8}$ | $4 \cdot 10^{-8}$ | $0.15–2.5 \cdot 10^{-7}$ | $10^{-4}$ |
| Spot size (μm) | 8 | 5 | 3–4 | 1 | 20 |
| Irradiance (W/cm$^2$) | $9.4 \cdot 10^4$ | [c]3–5 $10^6$ | [c]$2.5 \cdot 10^7$ | [c]$1–5 \cdot 10^{10}$ | [c]$3.2 \cdot 10^4$ |
| Fluence (J/cm$^2$) | $4.8 \cdot 10^2$ | $5–8 \cdot 10^{-3}$ | 1 | [c]$2.5 \cdot 10^3$ | 3.2[c] |
| nb of pulses | 1 | 3–5 | 1–2 | $2–5 \cdot 10^3$ | 5–8 |
| Total energy (J) | $2.4 \cdot 10^{-4}$ | [c]$0.3–1 \cdot 10^{-8}$ | [c]$1.4–2.5 \cdot 10^{-7}$ | $0.4–1 \cdot 10^{-1}$ | [c]$5–8 \cdot 10^{-5}$ |

*Because of imcomplete data in ref. 17, results of the nitrogen laser have been omitted.
[a]Data from two laser systems.
[b]Parameters for the drilling of human oocytes.
[c]Calculated from the available data.

proximately 27 μs. For irradiation times longer than 27 μs, heat diffusion out of the irradiated volume has to be taken into account in order to estimate the maximal temperature reached at the focus. Let us then estimate the time needed to heat, assuming adiabatic conditions, the volume in the immediate vicinity of the focus up to a temperature to 100°C, a temperature at which vaporization can start. Considering a specific heat of 4.2 J g$^{-1}$ K$^{-1}$ for water [29] and an initial temperature of 37°C, the energy density needed to heat water up to 100°C is 265 J/cm$^3$. At the 94-kW/cm$^2$ laser irradiance used, assuming again an absorption coefficient of 26 cm$^{-1}$, the laser power absorbed per unit of volume in the focal zone was 2.44 MW/cm$^3$. Thus the time needed to heat adiabatically the irradiated volume to 100°C is 108 μs.

As $\tau$ = 27 μs, the heat diffusion out of the irradiated volume cannot be neglected, and the effective temperature reached at the focus is below the adiabatic limit. Although the temperature reached in the ZP has not yet been experimentally determined, the absence of vapor bubbles indicates that it must lie below 100°C. As ZP lysis is induced, it must be high enough for protein denaturation to occur and is likely to be within 60 to 80°C.

A direct comparison of the physical processes at the origin of the ZP opening in our experimental conditions with that of other authors using completely different laser systems is difficult. This fact is due to the lack of reliable estimations for the laser energies effectively deposited in the target, as the absorption coefficients of the ZP at the investigated laser wavelengths are unknown. Despite this lack of information, a comparison of the laser irradiation conditions described so far in the literature was attempted and reported in Table 2. It is noteworthy that a wide range of irradiation conditions are applied with the various lasers in order to induce a comparable and quite specific effect on the ZP. Irradiance varies over 6 orders of magnitude from 32 kW/cm$^2$ up to 50 GW/cm$^2$. a range of 8 orders of magnitude can be derived from the published data for the total energy needed for ZP drilling. These puzzling discrepancies reflect, in part at least, the huge variations of light absorption by the ZP components in the investigated wavelength range and call for a more thorough investigation of the ZP optical properties.

In conclusion, the small-size 1.48-μm diode laser has been successfully integrated into an inverted microscope and provides a promising tool for the microdissection of subcellular targets. This wavelength can be focused through conventional culture medium and dishes and is extremely efficient in producing holes of controllable sizes in mouse ZP. The laser-target interaction process can be maintained within the millisecond range, with no optical readjustment during the drilling procedure, and performed in favorable non-touch conditions with virtually no mutagen or physicochemical hazards to the egg survival. The absence of deleterious effects induced by the laser drilling process is confirmed by the ability of drilled zygotes to develop in vitro similarly to controls.

The described set-up can be mounted into a compact device, which is well suited to fertility unit environments, and could be applied in the context of assisted procreation techniques.

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